



Studies on Germinal Center Biology

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STUDIES ON GERMINAL CENTER BIOLOGY

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A Thesis Submitted to the Faculty of
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for the Degree of Master of Medical Sciences in Immunology
Harvard University
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STUDIES ON GERMINAL CENTER BIOLOGY

Abstract

The germinal center reaction is at the heart of the humoral immune response; it comprises one major arm of the immune system, with the other arm being the cellular response. It is the site where B cells undergo somatic hyper-mutation, class switching, affinity maturation, and selection. The precise molecular regulation of germinal center development remains largely unknown. We isolated B cells from the mesenteric lymph node germinal center of a C57BL/6 mice and assessed their gene regulation programs by using ATAC-seq, a method for mapping open chromatin region, and RNA-seq, to determine global gene expression profiles with the aim of revealing new insights into the formation and maturation of germinal centers. We are also interested in the role of the germinal center in autoimmunity; using MRL/MpJ mice as a model, we found that there is an increase in 9-O-acetylation of sialic acid in MRL/MpJ germinal center B cells compared to C57BL/6 and BALB/c mice. MRL/MpJ mouse B-cells also show defect in BCR induced apoptosis. Ganglioside GD3 is a membrane-bound glycosphingolipid containing sialic acid, known to have a role in apoptosis induction by targeting the mitochondria. Interestingly, the pro-apoptotic effects of GD3 can be counteracted by 9-O-acetylation of the terminal sialic acid residue, giving 9-O-acetyl-GD3. We hypothesize that 9-O-acetylation of GD3 disrupts the apoptotic process of auto-reactive B cells in the germinal center, which ultimately results in autoimmunity.

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Chapter 1

Introduction

Initiation of the Germinal Center Reaction.

The germinal center (GC) was first characterized by the German biologist Walther Flemming in 1884 as a distinguishable micro-anatomical compartment in peripheral lymphoid organs. There Flemming observed most of the lymphocytes undergoing constant mitosis, leading him to hypothesize that the germinal center was the source of all lymphocytes in the human body¹. Much progress has been made in the field since then; we know now that the germinal center takes around a week to fully develop and is the site where B cells clonally expand, undergo somatic hypermutation in their immunoglobulin variable region gene, and undergo the selection required for high affinity antibody production.

T-cell dependent antibody responses occur when cognate T cells and B cells specific for an antigen interact at the inter-follicular region between B cell follicles and T cell zones, drawn to each other by changes in chemokine receptor expression. A subset of activated B cells then moves to extra-follicular foci (medullary cords), where they proliferate and differentiate into either short-lived plasma cells that secrete low affinity antibody, or un-switched memory B-cells². A second subset moves to B cell follicles to form secondary follicles; during this process, naïve B cells are pushed aside by the newly established germinal center and form the B cell mantle^{3,4}. The reasons behind B-cells choosing one fate or another remain unclear, but some studies

implicate as factors the strength of the B cell receptor (BCR) signal received from the foreign antigen, the amount of the antigen, and the degree of help received from T-cell^{5,6}.

Other studies show that antibodies secreted in the extra-follicular foci are of low affinity type and lack somatic mutations, whereas mutations occur at a much higher rate in germinal center B cells (termed somatic hyper-mutation), leading to affinity maturation. They also noticed that mutations were mostly clustered areas where antibodies came into contact with antigens. Interestingly, the germinal center does not appear to be absolutely necessary for affinity maturation, as germinal center-deficient mice show sensible affinity maturation when immunizations are introduced⁷. Germinal center B cells also express activation-induced deaminase (AID), the enzyme required for initiation of immunoglobulin gene somatic hyper-mutation and class switch recombination.

Germinal Center Formation and Establishment of Dark and Light zones.

In 1930 it was discovered that the germinal center is divided into two distinct regions, a darkly stained region with a high nucleus to cytoplasm ratio, and a lightly stained region occupied by follicular dendritic cells (FDCs) processes, aptly termed the dark zone and the light zone⁸. MacLennan in 1994 proposed a classical model of the germinal center in which the dark zone and the light zone are enriched by B cells called centroblasts and centrocytes, respectively. Centroblasts are large cells whose high mitotic figures indicate proliferation, lack surface immunoglobulin, and undergo

somatic hyper-mutation in the dark zone. Centrocytes, on the other hand, are smaller, non-proliferative, re-express surface immunoglobulin, and compete to bind antigens from the FDCs processes. The selected B cells then present antigens to follicular helper T cells (T_{fh}) in the light zone, where additional signals allow the B cells to survive and further differentiate into professional antibody secreting cells called plasma cells or memory B cells⁹. The movement between the two zones is primarily unidirectional from the dark zone toward the light zone.

As the field advances and new technology arises, some features are validated and others shown to be inconsistent with the classical model previously described. By using imaging studies such as *in vivo* real-time multi-photon microscopy studies and flow cytometric cell cycle analyses, it was found that the germinal centers B cells are in a highly motile and dynamic state, moving constantly between the dark zones and light zones in both directions. They are also shown to have dynamic interactions with both follicular dendritic cells and a subset of CD4⁺CXCR5⁺ helper T cells termed T follicular helper cells (T_{fh}).

The dark zone and light zones B cells, formerly known as centroblasts and centrocytes, respectively, have similar size and morphology based on forward-and side-scatter measurements. Using BrdU DNA synthesis-based cell proliferation assays¹⁰, cell proliferation was observed mainly in the dark zone but was also found to occur in the light zone, with similar numbers of cells in the S-phase cell cycle in both zones and a higher number of cells in the G2/M phase in the dark zone. The modern model also proposes that B cells selection events depend on T follicular helper cells rather than BCR signal strength, as the latter is more of a survival signal than a

selection limiting factor. This means that higher affinity B cells acquire more antigens from the FDC and present more peptide MHC (pMHC) molecules to T_{fh}, theorizing that B cells compete for T cell help instead of competing for antigen binding. The distinction between the dark zone and light zone phenotype is based on CXCR4, CD83, and CD86 expression. The dark zone is CXCR4^{hi} CD83^{lo} and CD86^{lo}, whereas the light zone is CXCR4^{lo} CD83^{hi} and CD86^{hi}. Microarray studies for gene expression showed that both zones have similar gene expression profiles, although proliferation and cell cycle genes are upregulated in the dark zone, and T cell help and activation genes show higher expression levels in the light zone^{11,12}.

Germinal center B cells differ from naïve B cell in several ways: 1) they are larger, 2) they demonstrate a dendritic morphology with leading edges and extended pseudopods due to their highly motile state, 3) they can be identified by increased expression of CD95 and the ligand of antibody GL7, 4) they bind to peanut agglutinin, 5) lack surface IgD, and 6) express higher levels of MHC II.

Molecular Control of Germinal Center

The molecular regulation of the germinal center reaction and dynamics is a major focus in immunology that has yet to be extensively studied. It remains unclear as to how an antigen-activated B cell decides whether to form the germinal center or differentiate into a low affinity extra-follicular foci plasmablast. Also unknown are the factors which cause a B cell to move between the dark zone and the light zone, as well as the decision to differentiate into a memory B cell or a high affinity plasma cell in the germinal center.

B cell lymphoma 6 (Bcl-6) is an important master transcriptional repressor that is primarily upregulated during the initiation of the germinal center reaction in both Tfh and germinal B cells. Its expression levels rise in Tfh one day after immunization whereas it is only detectable at low levels after two days in B cells. Without Bcl-6 the B cells fail to either enter the follicle or form the germinal center. Bcl-6 silences Bcl-2, an anti-apoptotic molecule that may play a role in preventing the formation and growth of self-reactive B cells resulting from somatic hyper-mutation. It also inhibits the DNA damage response by repressing the tumor suppressor p53 and ATR - a protein kinase involved in sensing DNA damage and activating the DNA damage checkpoints - and facilitates tolerance to frequent mutations. It also represses Blimp-1, which is required for plasma cell differentiation, and downregulates both BCR and CD40 signaling¹³. It is also noted that Bcl-6 deficient germinal center precursor B cells did not upregulate the expression of CXCR4, which is the chemokine receptor responsible for the migration of germinal center B cells to the dark zone.

Naïve B cells express Bcl-6 mRNA without translating any protein, which may suggest a role in post-transcriptional control of Bcl-6 expression in germinal center B cells and Tfh cells for faster upregulation of Bcl-6 protein expression upon activation^{14,15}.

Repression domain 2 (RD2) is an interesting region found in the Bcl-6 protein. Its presence is important for the migration of B cells toward the center of the follicles upon activation. Mutations in this domain will affect the expression of the G protein-coupled receptor Ebi2 – highly expressed on naïve but not germinal center B cells –

which must to be downregulated in order for the B cells to enter the center of the follicle.

Interferon-regulatory factor 4 (IRF4) is an intriguing transcription factor as it can regulate the formation of the germinal center by either activating or repressing Bcl-6 in a concentration-dependent manner. High levels of IRF4 will cause differentiation of plasmablasts by both activating BLIMP1 and repressing Bcl-6, while low levels will lead to the germinal center fate by activating Bcl-6. IRF4 deletion in B cells prevents germinal center formation after immunization¹⁶. A recent study from Cincinnati Children's Hospital Medical Center shows that IRF4 and IRF8 antagonize each other in a double negative feedback loop and regulate the bifurcation trajectories of activated B cells¹⁷.

Global analysis of genes from microarray data showed evidence of activation of early genes such as NF-kB, c-MYC, CD40, and BCR in the light zone, which indicate that B cell affinity based selection occurs in this compartment. c-MYC is expressed in early and late phases of the germinal center reaction, but is not expressed in dark zone B cells. It is believed that the late phase expression of c-MYC is responsible for B cell selection and cyclic re-entry and germinal maintenance. Inhibition of the MYC also leads to germinal center formation failure^{18,19}.

As previously mentioned, NF-kB is observed in subsets of light zone B cells. Studies have shown that CD40 activation leads to upregulation of IRF4 through NF-kB, which in turn represses Bcl-6 expression and ultimately leads to termination of the dark zone program. NF-kB is implicated in the maintenance and differentiation of

the germinal center, as deletion of downstream subunits of NF- κ B disrupts the germinal center reaction and arrests the generation of high affinity B cells²⁰.

As of the end of 2015, two studies describe Foxo1 and PI3K (phosphatidylinositol 3' OH kinase) as antagonistic master regulators of the germinal center^{21,22}. Foxo1 is highly expressed in the germinal center dark zone, and PI3K downregulates Foxo1 in the light zone where selection occurs.

Foxo1 (fork-head box protein O1) is a transcriptional factor that presents in the nucleus and regulate gene expression. Phosphorylation by protein kinase B (Akt) leads to its translocation to the cytoplasm, where it can then be ubiquitinated and degraded. A study has showed that germinal center-lymphomas carrying mutated versions of Foxo1 display resistance to Akt inhibition, providing strong support for the theory that Foxo1 plays a crucial factor in regulating gene expression within germinal center B cells.

We know that there are two major phases of B cell proliferation. Phase one occurs during the pre-B cell proliferation phase when PI3K activation of Akt can inhibit Foxo1, which in turn is necessary for pre-B cell proliferation in the bone marrow. A second phase of expansion occurs when mature B cells form the germinal center in response to antigen in a T cell dependent manner. The role of Foxo1 in mature B cells remains unclear.

In the mouse, Foxo1 deletion in germinal center B cells can lead to loss of dark zone phenotype cells and an inability to undergo antibody affinity maturation and class switch recombination. In addition, constitutive activation of the PI3K gene in

germinal center B cells causes a loss of the dark zone. Surprisingly, somatic hypermutation, cellular proliferation, and germinal center size are maintained; an unexpected and interesting phenomenon, as the dark zone has historically been characterized as the site that controls the cell cycle and germinal center B cell proliferation. Yet despite lacking the dark zone or dark zone phenotype cells, part of the dark zone program appears to remain intact and retains normal function as seen in normal germinal center B cells numbers in Foxo1-deficient mice. This lends yet more support to the argument that germinal center B cells proliferation can occur, to some extent, in the light zone¹⁰.

CXCR4 is another target gene of Foxo1 and is partly downregulated upon Foxo1 deletion or induction of PI3K activity. Loss of CXCR4 gives a similar phenotype to that of Foxo1-deficient mice, disrupts dark zone/light zone segregation, and prevents spreading of FDCs throughout the germinal center.

Using a bioinformatics approach, it was found that a large number of Foxo1-binding sites co-localize with sites bound by Bcl-6, which we know is a master transcriptional repressor important for the initiation of the germinal center. In addition, Blimp1, which encodes plasma cell differentiation, is co-repressed by Bcl-6 and Foxo1.

Epigenetic modification is one major aspect through which germinal center development and maturation can be influenced. The basic repeating structural and functional unit of chromatin is the nucleosome, which consists of ~146 bases of DNA wrapped around an eight-histone protein. Nucleosome-free regions, known as “open

chromatin regions”, are accessible to transcription factors and transcriptional regulatory machinery²³. Previous knockout mouse model studies have shown that removing certain transcription factors such as Bcl-6 renders the mice incapable of forming the GC or producing high-affinity antibodies²⁴.

We have mapped the chromatin accessibility and global gene expression of mouse germinal center B-cells isolated from mesenteric lymph nodes and the spleen. We hypothesize that identifying these regions across the genome will allow us to identify motif enrichment of transcription factor binding sites and gain new insights into the formation and maturation of germinal centers as well as the transcription factors involved in the process.

Germinal Center B-cell and Autoimmunity.

We are also interested in the role of germinal center in autoimmunity. Auto-reactive B cells can arise from germinal center dysregulation and failure of certain checkpoints, with the results of autoantibodies being produced by plasma cells. MRL/MpJ mice is the parent and control strain for MRL/MpJ-*Fas Ipr*, it exhibit autoimmune disorders and can be used to study the phenomena.

Sialic acid is generic term for N- or O- substituted derivatives of neuraminic acid, a sugar with 9-carbon backbone²⁵. N-acetylneuraminic acid (Neu5Ac or NANA) is the most common member of this group. In animal, sialic acids are attached to the ends of glycan chains and can be expressed on the cell surface as components of a class of glycolipids called gangliosides or glycoproteins. 9-O-acetylation is a common

modification of sialic acids, it's been observed in different cell types. GD3 is a ganglioside, which is a membrane bound glycosphingolipid containing sialic acids linked to sugar chain. It has been shown to be involved in Fas-mediated apoptosis in hematopoietic cells²⁶. Interestingly, it has been shown that 9-O-acetylation modification of GD3 can reverse its apoptotic effects, suggesting a way for cells to avoid the fate of GD3-induced apoptosis^{27,28}.

Previous studies showed that MRL/MpJ B-cells are protected from apoptosis and our lab showed that those B-cells have high 9-O-acetyl sialic acid. We looked at germinal center B-cells in MRL/MpJ mice and found that they have increased 9-O-acetylation level. We propose that 9-O-acetylation of GD3 disrupt the apoptosis process of auto-reactive B cells in the germinal center which lead to autoimmunity.

Chapter 2

Short Introduction

Germinal centers are specialized sites where mature B-cells undergo clonal expansion, somatic hypermutation, class switching and affinity maturation, leading to the generation of high-affinity antibodies. However, the exact molecular mechanism by which the germinal center form remains unknown. The transcription factor Bcl-6 was discovered to be essential for the germinal center formation and without Bcl-6 B-cells are incapable of forming the germinal centers. Bcl-6 protein is highly expressed in germinal center B-cells compared to naive B cells. Since, Bcl-6 is a master transcriptional repressor, therefore we hypothesize the presence of other transcription factors playing a major role in the development and maturation of the germinal center. By taking epigenetics approach, we are using ATAC-seq to look at the open chromatin regions for motif enrichments of transcription factors binding sites in germinal center B-cells from mesenteric lymph node and spleen of B6 mice and comparing them to naive B-cells. We are using RNA-seq also to look at the global gene expression profile and whether those open regions are actively being transcribed. We believe using these next generation sequencing technology, we can unravel new insights into the formation and maturation of germinal centers and the epigenetic regulators involved in the process.

We are also interested in the germinal center role in autoimmunity in MRL/MpJ mice. The generation of auto-reactive B cells is an inevitable by-product of repeated round of mutations in the germinal center dark zone and aberrant selection of these

auto-reactive B cells in the light zone can arise from germinal center dysregulation and failure of certain checkpoints, with the results of autoantibodies being produced by the progeny long-lived plasma cells causing autoimmune diseases. GD3 is a ganglioside playing major role in CD95-induced apoptosis and 9-O-acetyl modification of its sialic acids suppresses the pro-apoptotic effect. MRL/MpJ mice are protected from apoptosis and we found there is an increased level of 9-O-acetylation in MRL/MpJ germinal center B-cells compared to B6 and Balb/c mice. We propose that 9-O-acetylation of GD3 disrupt the apoptosis process of auto-reactive B cells in the germinal center which lead to autoimmunity.

Materials and methods

Mice. 20 weeks old male C57BL/6 (B6) Balb/c mice and MRL/MpJ were obtained from the Jackson Laboratory and used in this study. Animal procedures were approved by the animal research care at the Ragon Institute of MGH, MIT and Harvard.

Antibodies, staining, flow cytometry and cell sorting. The following murine monoclonal antibody conjugates were used: Pacific Blue (PB)-RA3-6B2 (anti-CD45R/B220, Rat IgG2a, κ), PerCP/Cy5.5-6D5 (anti-CD19, Rat IgG2a, κ), Brilliant Violet 510-11-26c.2a (anti-IgD, Rat IgG2a, κ), FITC-RMM-1 (anti-IgM, Rat IgG2a, κ), PE-90 (anti-CD38, Rat IgG2a, κ), all from Biolegend. PE-Cy7-Jo2 (anti-CD95, Armenian Hamster IgG2, λ 2) from BD biosciences, and eFlour660-GL7 (anti-GL7) from eBioscience.

Single cell suspensions were made from mesenteric lymph node and spleen by mechanical disruption on 70-mm nylon cell strainers (Falcon) in Dulbecco's Modified Eagle's medium (DMEM), then were washed and kept on ice. Red cells were lysed with 1 ml ACK lysing buffer. The lysis buffer was neutralized by adding 10 ml PBS and 0.2% BSA. Before staining, 1×10^6 cells were reacted with 2.5μ of anti-CD16/CD32 (Fc γ III/II receptor), rat IgG_{2b}, κ ; BD). Surface staining was performed using appropriate dilution of antibodies in 12 x 75-mm polystyrene round bottom tubes in a volume of 100μ for 30 min in the dark at 4° C.

Flow cytometry analysis was performed on 4 Laser LSR 2 (4R). Unstained cells and beads were used to set voltage and single color positive controls were used for

electronic compensation (Beckman Coulter). Processed samples were analysed using FloJo analysis software.

For cell sorting, cell suspensions were first prepared from mesenteric lymph node and spleen in Dulbecco's Modified Eagle's medium (DMEM). Cells at density of 5×10^6 cells/ml were labeled for 30 mins on ice with the same antibodies used in flow cytometry analysis.

B cell purification. Spleens were mechanically dissociated on 70-mm nylon cell strainers (Falcon) in Dulbecco's Modified Eagle's medium (DMEM), then were washed and kept on ice. Red cells were lysed with 1 ml ACK lysing buffer. B-cells were labeled for 30 minutes on ice with biotin conjugated anti-B220 (BD Pharmingen), then were washed with MACS running buffer, and then were labeled for 30 minutes with streptavidin microbeads (Miltenyi Biotec). Samples were depleted of unlabeled cells by autoMACS according to the manufacturer's instructions, resulting in a B-cell purity of at least 90% as determined by flow cytometry.

RNA-Seq. To profile global gene expression. 10^3 - 10^5 cells from each population were sorted into RLT + 1% BME lysis buffer (Qiagen). We used RN1easy total RNA isolation and cleanup with optional DNase treatment from W.M. Keck foundation biotechnology resource laboratory at Yale university protocol. We used SMART-Seq protocol for RNA-seq developed by Aviv Regev laboratory.

ATAC-Seq. To profile open chromatin. 10^4 to 50^4 cells from each population were sorted into DMEM. Cells were lysed first then transposition reaction and purification occur with insertion of adaptors that can be used to amplify the sample. During PCR amplification barcoded PCR primers are used. ATAC-seq protocol used is developed by Buenrostro et. al (2013).

Preparation of CHE-Fc/CHE-FcD Chimera. HEK 293 CHE-Fc stably transfected cells were obtained from Pascal Crottet (J.Biol. Chem. 2002). DFP (Diisopropylfluorophosphate, Sigma D0809) added to the CHE-Fc prep and left on ice for 30 mins. The DFP will begin breaking down in the aqueous solution.

Results

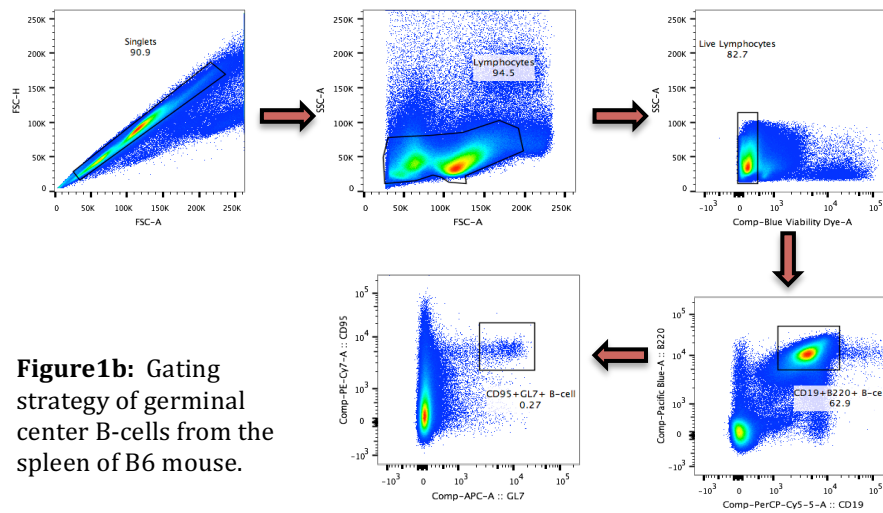
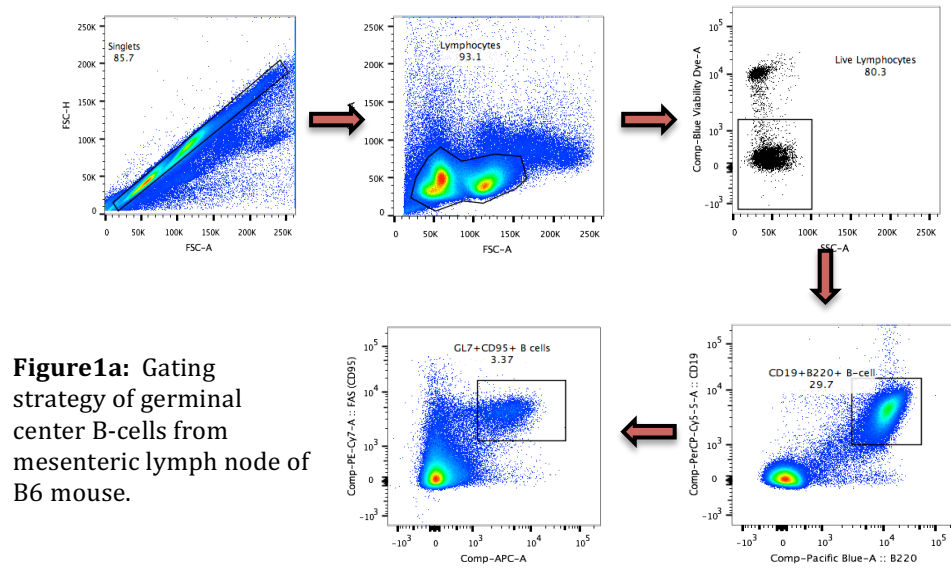
Mesenteric Lymph node forming germinal center without immunization.

The gut immune system is daily exposed to plethora of antigens, mostly in the form of food antigens and enormous numbers of commensal microbes. Commensals generally involved in symbiotic interactions with the host. So, the surface of the gut mucosa is constantly facing antigenic stimuli. Secondary lymphoid tissue, such as Peyer's patches, and mesenteric lymph node are able to respond to antigenic stimuli releasing cytokines or producing antibodies (secretory IgA).

The mesenteric lymph nodes, of lengthened shape, that lies between the mesentery membranes, close to the ascending portion of the colon. Is a key site for tolerance induction to food antigens and the draining lymph nodes for the gut. Dendritic cells are present in the mucosa underlying the epithelium and can take up antigens from foods or commensals. They take these antigens to the draining mesenteric lymph node, where they present them to naive T-cells or B-cell²⁹. The germinal centers are formed in the follicles without the need for immunization and can be used as a tool to study the reaction with expected bias toward IgA class switch.

We took the mesenteric lymph node and the spleen from 20 weeks old male C57BL/6 (B6) mice. There were $\sim 15 \times 10^6$ cells in the mesenteric lymph node and $\sim 75 \times 10^6$ cells in the spleen. Both of them are stained them with germinal center and B-cell markers. Germinal center B-cells are CD19⁺ B220⁺ GL7⁺ and CD95⁺. By using flow cytometry we identified the germinal center B-cells in the mesenteric lymph

node and spleen (figure 1a and 1b). Germinal center B-cells were higher in mesenteric lymph node than the spleen.



Specimen_001_Spleen.fcs
CD19+B220+ B-cell
4.75E5

Open Chromatin regions of the germinal center B-cells.

The fundamental unit of chromatin, the nucleosome, organizes ~147 base pairs of DNA turn around an octamer of four core histones: H2A, H2B, H3, and H4. Small sections of DNA called linker DNA serve to join the nucleosomes together, and these are associated with the linker histone H1. Nucleosome-free regions, known as “open chromatin,” contain regulatory elements—such as promoters and enhancers—that play a critical role in gene regulation³⁰.

When the DNA is packed in a heterochromatin state (closed chromatin), the nucleosome prevents the transcriptional machinery from accessing the DNA sequences or at least make it difficult to ^{happen}. To solve this problem, certain histones and DNA modification occur to expose key regions of the DNA (open chromatin) required for interaction with proteins controlling transcription and maintenance of the genome. Regulatory areas in DNA sequences which control gene function and expression mostly in the region of low nucleosome occupancy. Transcription factors and other transcriptional regulatory protein bind to gene promoter sites, and these sites of DNA usually have low number of nucleosome than the adjacent coding sequences^{31,32}.

Cellular behavior and phenotype are mostly controlled by epigenetic modification that regulate the compaction and nucleo-protein structure of chromatin. By using epigenetic studies approach, lots of information can be provided like transcription

factors binding sites, the position of nucleosome and chromatin accessibility at regulatory regions such as promoters and enhancers.

We used an assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq), a method for mapping chromatin accessibility genome-wide. It probes DNA accessibility with hyperactive Tn5 transposase, which simultaneously cut and paste adaptors into the open chromatin regions. Then the adaptors can be used to amplify the sample using barcoded primers, for high throughput sequencing at regions of increased accessibility to map regions of transcription factors binding and nucleosome position. The advantages of ATAC-seq over other epigenetic assays are: can be done with low cell numbers, as low as 50,000 cells, and it only requires 3 hours in total³³.

Thirteen 20 weeks old male C57BL/6 (B6) mice were used, mesenteric lymph node and spleen were stained for germinal center B-cell and naive B-cell. Specific cells are sorted out then ATAC-seq protocol were carried out on fresh unfixed cells for maximum efficiency.

Open chromatin regions were identified on those samples, using the software MACS2 (Model-based Analysis of Chip-Seq)^{34,35}. MACS2 first calculates a “background signal” based on the genome-wide mapping of ATAC-seq reads and then identifies “peaks,” or regions in the genome where reads map more densely than they do across the whole genome. These densely-mapped reads have the sequences of the fragments captured from the tagmentation of open chromatin, and the reads that map to a particular region can be pieced together to form what appears to be a pointed

“peak”. The peak “summit” is the point in the interval that corresponds to the start site of the most number of reads that comprise the peak.

The “height” of the peak indicates the number of reads that map to a region, and a peak that is higher than the background implies that its interval was exposed enough to be fragmented and tagged.

Signal transducer and activator of transcription 3 (STAT3), is one example of chromatin region we found so far. Enrichment alignment of reads “peaks” in STAT3 region were called, in germinal center B-cell samples and not on naive B-cells (figure 2). STAT3 is transcription factor and member of the STAT protein family. It has a major signaling role in germinal center B-cell and T follicular helper cell differentiation³⁶. STAT3-deficient mice show fewer germinal center B-cells in the late phase of germinal center formation, plasma cell differentiation and antibody production were impaired also.

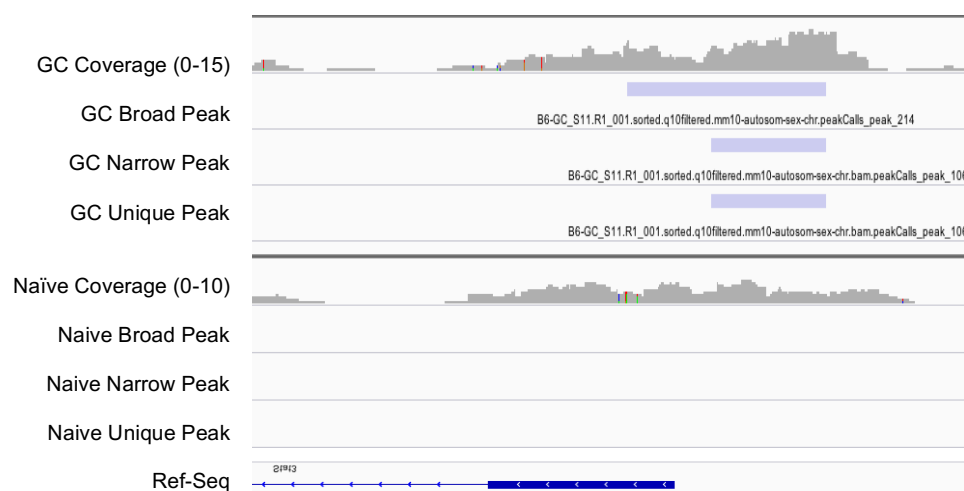


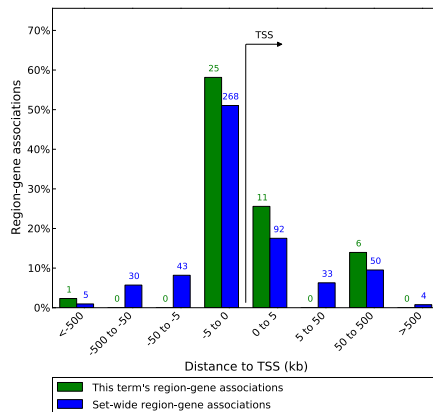
Figure 2: Example ATAC-seq coverage: STAT3 on the Integrative Genomics Viewer (IGV).

We also looked at the location of germinal center B-cell peaks relative to transcription start site (TSS) in different signaling pathways using the GREAT (Genomic Regions Enrichment of Annotations Tool) software. It assigns biological meaning to a set of non-coding genomic regions by analyzing the annotations of the nearby coding genes. Thus, it is particularly useful in studying cis functions of sets of non-coding genomic regions³⁷. In summary, the purpose is to predict possible transcriptional regulatory roles of the peaks identified in ATAC-seq.

We found peaks near the transcription start site (TSS) of c-MYC, CHEK2 and BRCA (figure 3a, 3b and 3c). c-MYC is regulator gene that codes for a transcription factor, has a role in cell proliferation, differentiation, apoptosis and cell growth. It expressed in early and late phases of the germinal center reaction, but is not expressed in dark zone B cells. It is believed that the late phase expression of c-MYC is responsible for B cell selection and cyclic reentry and germinal maintenance. Inhibition of the MYC also leads to germinal center formation failure^{18,19}.

CHECK2 is Checkpoint kinase 2, is tumor suppressor gene. Has role in DNA repair, cell cycle arrest or apoptosis in response to DNA damage. BRCA1 is breast cancer type 1 susceptibility protein, also a tumor suppressor gene that has a role in DNA repair process. With the repeated mutations and cellular proliferation that happen in the germinal center, the activation of those genes in germinal center B-cells is expected.

Peaks near TSS's of c-Myc target genes



Peaks near TSS's of of CHEK2-PCC target genes

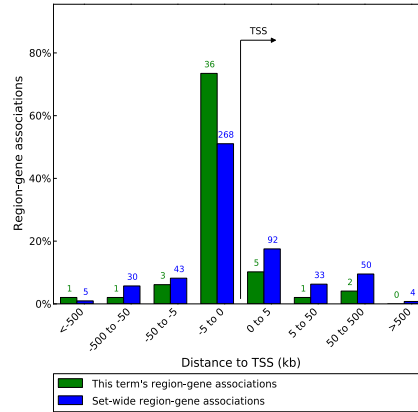
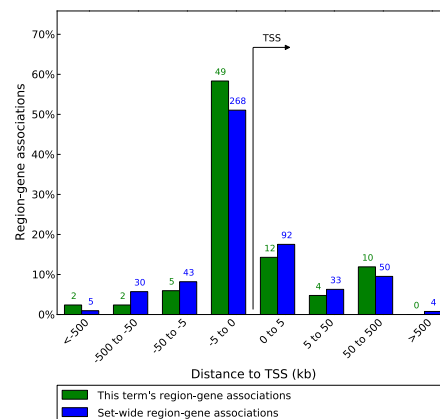


Figure 3: Location of GC B-cell peaks relative to TSS in different signaling pathways. Blue: location of peaks relative to TSS's of all gene sets associated with open chromatin; Green: location of peaks relative to TSS's of specific gene sets associated with open chromatin. The purpose is to predict possible transcriptional regulatory roles of the peaks identified in ATAC-seq. The GREAT Software (from Gill Bejerano's lab at Stanford) was used to identify these regions.

Peaks near TSS's of genes in the BRCA1-PCC



Motif enrichment for transcription factors binding sites is another information we can look at from ATAC-seq data using HOMER (Hypergeometric Optimization of Motif EnRichment). HOMER is suite a tools for motif discovery and next-gen sequencing analysis using algorithm to find 8-20 bp motifs in large scale genomics data. Sp1, Klf4 and klf5 binding site among others were found enriched (figure 4).

Sp1 is zinc finger transcription factor that bind to GC rich motifs of many promoters, has role in cell differentiation, cell growth, apoptosis and immune response. Sp1 and pax5 mediate activation of germinal center B-cell specific m17 promoter, M17 gene involved in signaling transduction³⁸. The Kruppel-like family of transcription factors (KLFs) are also a set of zinc finger DNA-binding proteins that regulate gene expression.

Figure 4: Motif Enrichment in GC-exclusive accessible chromatin. HOMER (Hypergeometric Optimization of Motif EnRichment) us used. Sp1, Klf4 and klf5 binding site among other were found enriched

Rank	Motif	Name	P-value	log P-value	q-value (Benjamini)	# Target Sequences with Motif	% of Targets Sequences with Motif	# Background Sequences with Motif	% of Background Sequences with Motif
1		Sp1(Zf)/Promoter/Homer	1e-66	-1.538e+02	0.0000	195.0	53.72%	7041.9	14.63%
2		KLF5(Zf)/LoVo-KLF5-ChIP-Seq(GSE149402)/Homer	1e-49	-1.134e+02	0.0000	253.0	69.70%	15214.6	31.60%
3		NPY(CCAAT)/Promoter/Homer	1e-33	-7.729e+01	0.0000	112.0	30.85%	4076.5	8.47%
4		KLF4(Zf)/uES-KLF4-ChIP-Seq(GSE11431)/Homer	1e-31	-7.325e+01	0.0000	109.0	30.03%	4047.0	8.41%
5		GFY-Sta(1,Zf)/Promoter/Homer	1e-11	-2.687e+01	0.0000	23.0	6.34%	459.1	0.95%
6		GFY(1)/Promoter/Homer	1e-11	-2.574e+01	0.0000	23.0	6.34%	486.5	1.01%
7		ELF1(ETS)/Junkat-ELF1-ChIP-Seq(SRA014231)/Homer	1e-9	-2.284e+01	0.0000	80.0	22.04%	5042.5	10.47%
8		GABPA(ETS)/Junkat-GABPA-ChIP-Seq(GSE17954)/Homer	1e-9	-2.168e+01	0.0000	84.0	23.14%	5555.8	11.54%
9		ETS(ETS)/Promoter/Homer	1e-8	-1.929e+01	0.0000	55.0	15.15%	3104.2	6.45%
10		EBF1(ETS)/HeLa-EBF1-ChIP-Seq(GSE11477)/Homer	1e-8	-1.893e+01	0.0000	80.0	22.04%	5488.7	11.40%

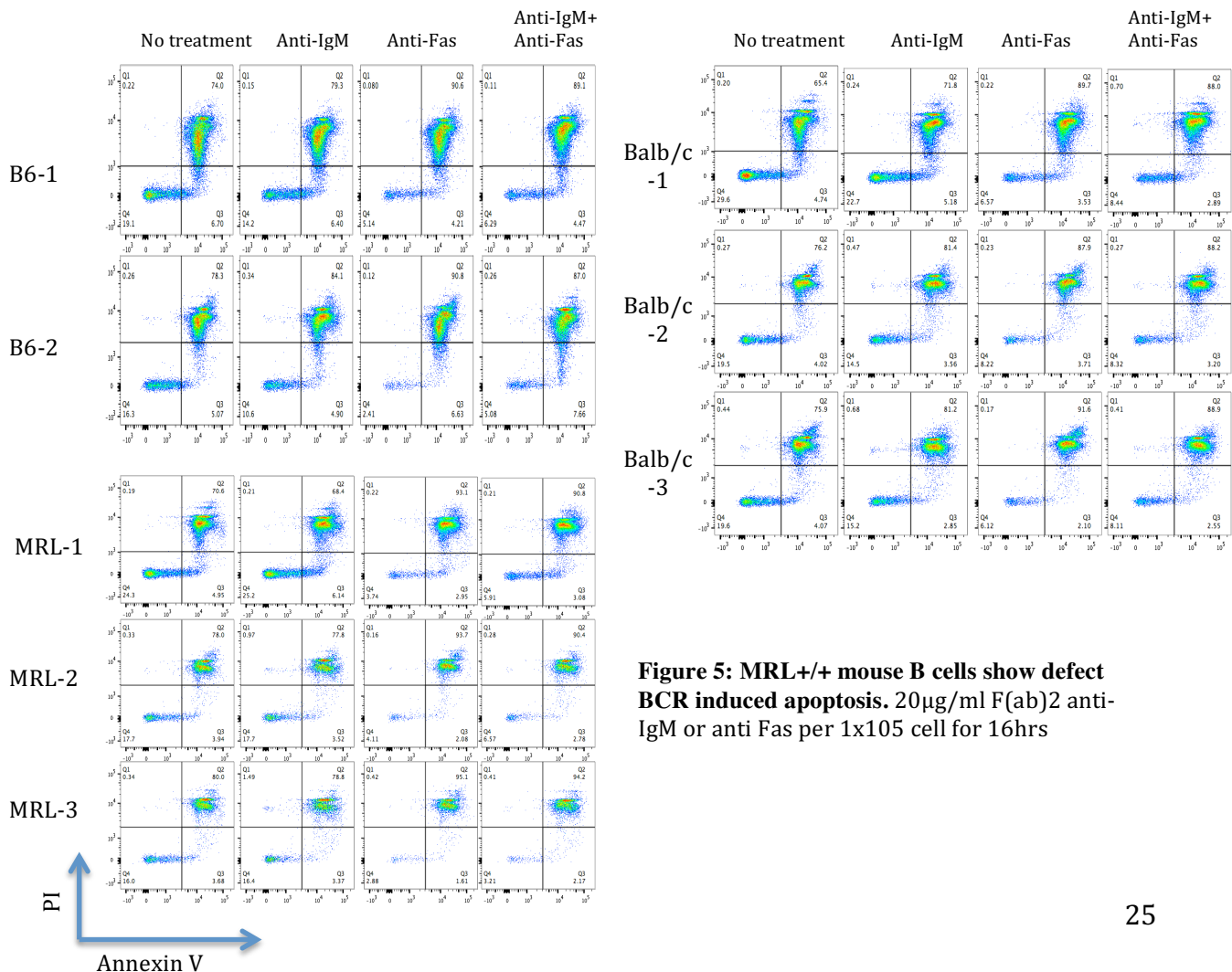
B-cells and Autoimmunity.

Autoimmunity is the presence of self-reactive immune response. This immune mediated response can be driven by T-cells or auto-antibodies secreted by the progeny of B-cells plasma cells, either by binding to self-antigens or by forming immune complexes. These self-reactive cells are usually eliminated prior to becoming active within the immune system by different checkpoint mechanisms. Any abnormalities in these regulations can result in escaping of these auto-reactive cells, which can lead to autoimmune mediated injury^{39, 40}.

Most injury causing autoantibodies that bind antigens with high affinity, are IgG antibodies which undergone rounds of mutations that are selected by antigen⁴¹. And most of those antigens are protein. These are classical features of antibodies generated from the germinal center by plasma cells, terminally differentiated cells from B-cells in the light zone after multiple round of mutations in the dark zone.

B-cells undergo multiple tolerance checkpoints during development. Our focus is on germinal center B-cells abnormalities and the generation of auto-reactive antibodies. Many strong evidences suggested that that FDC-derived signal, BCR signals and Tfh cell-derived signals all participate to germinal center B-cell survival, proliferation, differentiation and selection, but the precise order and the molecular elements of the cellular interaction are still not clear. Low affinity or self-reactive B-cells are eliminated by apoptosis.

Here we used the MRL/MpJ mice, which are the parent and control strain for for MRL/MpJ-*Fas*^{lpr}. Despite carrying the normal *Fas* gene, MRL/MpJ mice also exhibit autoimmune disorders, but symptoms are manifested much later in life compared to those the MRL/MpJ-*Fas*^{lpr} mice. As a strain developed as the control for MRL/MpJ-*Fas*^{lpr}, MRL/MpJ mice are useful in the study of their comparable defects and diseases, including systemic lupus erythematosus, Sjorgren syndrome and other autoimmune diseases. We first looked at MRL/MpJ B-cells apoptosis by inducing either BCR signaling or through CD95 ligation or both, and compare the result with B6 and Balb/c mice. We found there is a defect in BCR induced apoptosis process in MRL/MpJ mice.



It is known that GD3 is a ganglioside playing major role in CD95-induced apoptosis and 9-O-acetyl modification of its sialic acids suppresses the pro-apoptotic effect. So, we looked at level of 9-O-acetylation in MRL/MpJ germinal center B-cells compared to B6 and Balb/c mice. By using (CHE-FcD) influenza C virus hemagglutinin-esterase which is a membrane-bound glycoprotein that binds specifically to 9-O-acetylated sialic acids treated with DFP (Diisopropyl fluorophosphates) to abrogate the esterase activity, we found an increase in 9-O-SA acetylation in germinal center B-cells in MRL/MpJ mice comparing to B6 and Balb/c mice. We propose that 9-O-acetylation of GD3 disrupt the apoptosis process of auto-reactive B cells in the germinal center which lead to autoimmunity.

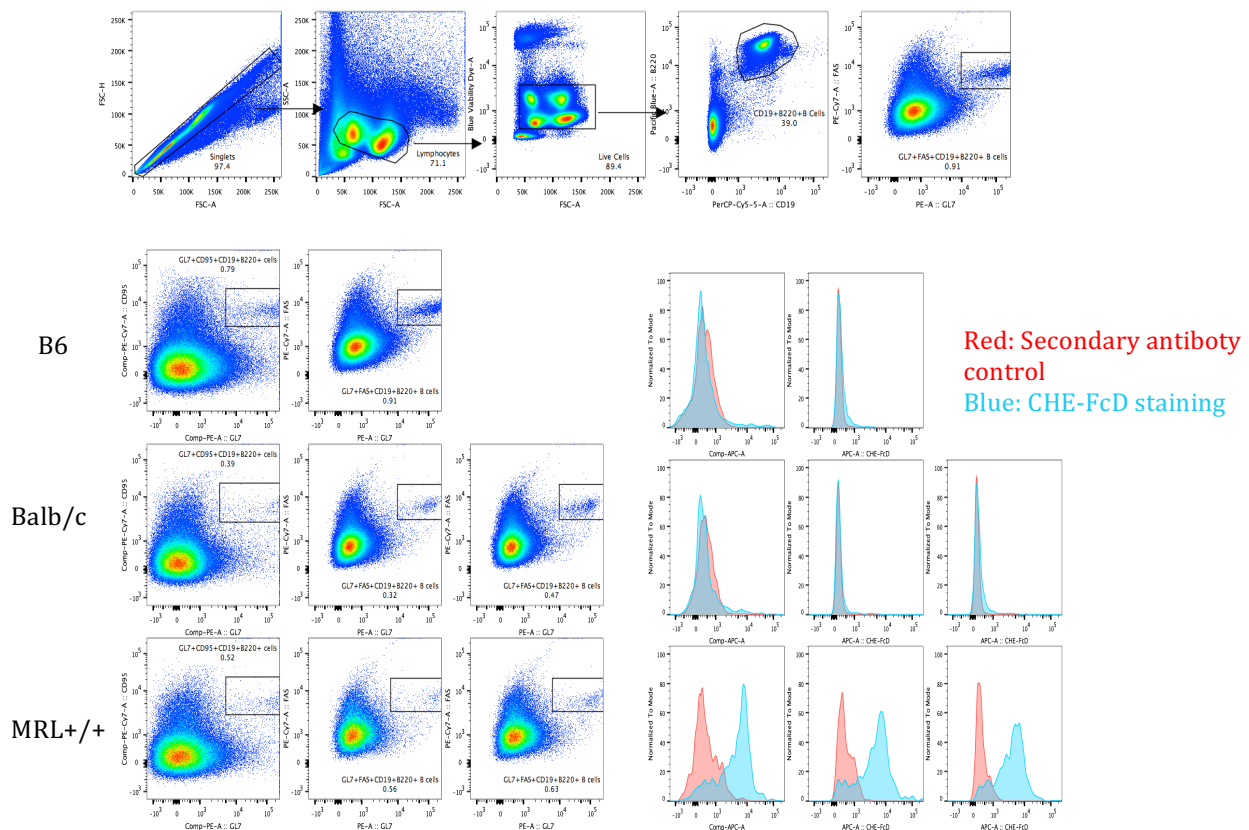


Figure 6: MRL+/+ mice have normal Germinal center B cell numbers but high 9-O-SA-Acetylation

Brief Discussion.

We demonstrate that mesenteric lymph node is resourceful site for germinal center reaction without the need for immunization, due to the continuous exposure to the food antigens and gut commensals.

B6 mice were used and the mesenteric lymph node and spleen were stained using antibodies that are specific for surface markers for germinal B-cells. And by using ATAC-seq, an epigenetic assay, we were able to access the open loci regions and data were generated. We found a couple regions that are specific for germinal center B-cells were opened like STAT3, also peaks near certain transcription start site were noticed for factors that play roles in cell differentiation, proliferation and apoptosis. Some Motif analysis were done also, looking at transcription factors binding sties

We demonstrated that this method is a robust and sensitive epigenomic profiling tool that can provide an multi-dimensional view for trancriptomic and epigenomic gene regulation especially when is done with RNA-seq and Chip-seq.

Several molecular mechanisms have been proposed to explain the compelling evidence for the germinal center origin of many autoantibodies. Our finding suggested an indirect link between 9-O-acetylation of sialic acid in the germinal center B-cells and the process of apoptosis, leading to autoimmunity. Further tests needed to be done to explore the different aspects involved in the process.

Chapter 3

Discussion and Perspectives.

Assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq), is a epigenetic assay for mapping chromatin accessibility and open loci genome-wide. This when combine with global gene expression profiling (RNA-seq) can give us a powerful tool that help us to elucidate many fundamental questions and yet still vague areas regarding the molecular control that determine germinal center B-cell fate and how these factors regulate each other. How do multiple molecular factors work together to regulate the expression of germinal center B-cell developmental genes? Also what make an activated B-cell decide to enter the germinal center pathway instead of differentiation into short-lived plasmablasts, what are the molecular cues that drive a cell to recirculate into the dark zone and how and when is the decision to undergo class switch recombination to a certain isotype determined. And finally what sequence of events signals a germinal center B-cell to cease cyclic re-entry and to terminally differentiate to memory B-cell or long lived plasma cells?

Our results strongly indicate that we can track down those open chromatin regions in certain B-cell developmental stage like germinal center B-cells here and compare it with naïve B-cell stage. Peaks were called and certain possible regulatory factors were found; yet tests need to be repeated. We still need to find out also how accessibility of regulatory chromatin predicts transcriptional profile.

We also immunized B6 mice with ovalbumin and using aluminium phosphate salt as an adjuvant. Germinal centers B-cell perfectly formed and were sorted out between day 10 to day 13, and ATAC-seq and RNA-seq data yet still to be analyzed.

We are planning also planning to test the epigenomic and transcriptomic profile in a more controlled environment by using B1-8 mice developed by Klaus Rajewsky, whose BCRs have a higher affinity for NP and transferred those B1-8 cells to OT-II mice which bind specifically to Ovalbumin and then immunize the mice with NP-OVA immunogen and track down germinal center reaction B-cells. In this case we will have a T-cell specific for ovalbumin and B-cell specific for NP. And analyze their epigenomic and transcriptomic profile. Deep sequencing analysis need to be done also like identifying super enhancer regions, which are peaks called on ChIP-seq data for cell type-specific master transcription factors. Identifying those regions will help us to understand more about the regulatory elements and their roles in cellular faith decisions.

Expression sequence pattern and functional requirement of molecules during the different phases of germinal reaction is essential and need to be looked at too. From previous data we know that germinal center reaction has three separate phases during the first 7 days, the early initiation, late initiation and proliferation and establishment phase. Different regions are opened in those different stages and whether those open regions are being actively transcribed or just playing a regulatory role by having direct to indirect effect in the transcription factors needed to be investigated.

Single cell RNA-seq will be an important tool for us to investigate the dark zone and light zone germinal center cells and the differentiation into memory B-cells or plasma cells. And finally genome editing using CRISPR-Cas9 knockouts to validate the function of promising hits is essential.

Considerable progress has been made in understanding in and B-cell and their role in autoimmunity. We now have a greatly outlined picture of different checkpoints events during B-cell developments, and germinal center dysregulation and the generation of aberrant autoantibodies is an important part of understands autoimmune diseases. The interference of apoptosis process in the germinal center will lead escaping of the self-reactive B-cells that suppose to die and the generation of autoantibodies. It was suggested that T_{fh} cell had a major role in the negative selection of those cell by ligating CD95, which is highly expressed in germinal center B-cells. And as we mentioned earlier GD3 is a ganglioside that has major role in CD95-induced apoptosis and 9-O-acetyl modification of its sialic acids suppresses the pro-apoptotic effect We found that 9-O-Acetylation of sialic acid is increased in germinal center B-cells, and we are planning to remove the 9-O-Acetylation by taken advantage of the esterase activity in CHE-Fc without the DPF in MRL/MpJ mice and look at the apoptosis process if its back to normal. Also by using certain biochemical assays to remove glycoproteins by using trypsin or removing lipids using methanol to validate GD3 roles in the process.

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